PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Vinson et al.

Group Art Unit: 1636

Application No. 10/059,720

Examiner: Quang Nguyen

Filed: January 29, 2002

For: EXTENSION OF A PROTEIN-PROTEIN INTERACTION SURFACE TO INACTIVATE THE FUNCTION OF A CELLULAR PROTEIN

DECLARATION UNDER 37 C.F.R. § 1.132 OF CHARLES R. VINSON, PH.D.

Mail Stop Non-Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Charles R. Vinson, hereby declare that:

1. I am a coinventor of the subject matter disclosed and claimed in the above-identified patent application.

2. I am a Principal Investigator in the Laboratory of Metabolism at the National Cancer Institute, Center for Cancer Research, at the National Institutes of Health. I have authored or co-authored numerous scientific journal articles, which are listed in my curriculum vitae, which is attached hereto as Exhibit 1.

3. Our laboratory, in collaboration with other groups, has successfully created numerous transgenic mouse models that express dominant negative, acidically modified nucleic acid binding proteins (such as A-CREB and A-C/EBP) according to the methods described in the above-identified application.

4. One such transgenic mouse model expressed A-FOS, a dominant negative, acidically modified Fos molecule that inhibited AP-1 DNA binding (see Olive et al., *J. Biol. Chem., 272(30),* 18586-18594 (1997), attached hereto as Exhibit 2) under the control of the tetracycline operator region (Tet-O-A-FOS). A-FOS was produced by fusing an acidic extension with the leucine zipper dimerization domain of FOS, as described, for example, at page 53, paragraph [0131], and Figures 6-8, of the above-identified application.

5. The tetracycline operator driven A-FOS transgene construct contained the 7x tetracycline repressor operator system, the CMV Minimal Promoter, a Hemagglutininin epitope, A-FOS cDNA from the CMV566 plasmid constructs (see, e.g., Example 4 of the above-identified application; and Olive et al., *supra*), and a SV40 polyA fragment containing the small T-antigen intron that preceded the polyA site obtained from the plasmid pRSVNeo (Gorman et al., *Mol. Cell Biol., 2,* 1044-51 (1982)).

6. To create the transgenic mice, linearized tetracycline operator driven A-FOS transgene construct was injected into mouse pronuclei by standard procedures. The founder pups were screened by Southern hybridization of their tail DNA preparations using the linear tetracycline operator driven A-FOS transgene construct as a probe, and PCR for a 500 bp region spanning the tet-operon region into the A-FOS cDNA.

7. The A-FOS transgenic mice were then crossed with the bovine Keratin 5 promoter tet-transactivator mouse (bK5-tTA; Diamond et al., *J. Invest. Dermatol., 115,* 788-94 (2000); and Liu et al., *Proc. Natl. Acad. Sci. USA, 98,* 9139-44 (2001)), which tissue-specifically targeted expression to basal keratinocytes and out rooth sheet hair follicle cells (Ramirez et al., *Differentiation, 58,* 53-64 (1994)). Targeted gene expression previously had been demonstrated to be regulated by oral or topical administration of tetracyclines (Diamond et al., *supra*).

8. To demonstrate the functional inhibition of AP-1 DNA binding and transactivation, double transgenic (K5/A-FOS) mice were crossed to an AP-1 reporter transgenic line, TRE-Luciferase (Rincon et al, *Embo. J., 13,* 4370-81 (1994)), and subjected to a multiple stage skin carcinogenesis. Triple transgenic mice, and control animals (i.e., A-FOS/TRE double transgenic mice, TRE-Luciferase mice, or wild-type mice) were painted with the phorbol ester PMA on one ear for 6 hours. PMA induced a strong response in control TRE-Luciferase mice following PMA application relative to wild type samples. This PMA induction of luciferase was completely blocked with the expression of the A-FOS transgene in the triple transgenic mice and

A-FOS/TRE double transgenic mice. These results were corroborated by analysis of primary keratinocytes from TRE-Luciferase mice and the triple transgenic mice following PMA treatment for 6 hours. Strong induction of luciferase activity (indicating AP-1 binding and transactivation) was observed following PMA treatment in the TRE-Luciferase mice, but not in the triple transgenic mice. These results demonstrate that the A-FOS transgene functionally blocks transactivation through inhibition of AP-1 complex binding to DNA.

9. Epidermal carcinogenesis is strongly regulated through AP-1 activity as was shown previously by analysis of the c-fos null mice and the Jun transactivation domain dominant negative (TAM67) that had been targeted to basal keratinocytes (Saez et al., *Cell, 82,* 721-32 (1995); Watts et al., *Mol. Carcinog., 13,* 27-36 (1995); and Young et al., *Proc. Natl. Acad. Sci. USA, 96,* 9827-32 (1999)). Studies previously demonstrated that AP-1 factors are critical for metastatic conversion and to some degree tumor incidence (Saez et al., *supra*; and Young et al., *supra*). Following a multi-stage skin carcinogenesis protocol, A-FOS expressing mice (i.e., double transgenic K5/A-FOS mice) did not produce papillomas or carcinomas as observed in control littermates. These mice instead developed small benign sebaceous adenomas. When papillomas were produced prior to A-FOS expression, progression to malignant carcinomas was prevented, and a trans-differentiation into benign sebaceous adenomas was observed. These results indicate that A-FOS expression inhibits and treats squamous tumors through inhibition of tumor progression from benign to malignant states, as well as a differentiation towards a less malignant phenotype.

10. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 3/3/04

Charles R. Vinson, Ph.D.

CURRICULUM VITAE

Name: Charles Robert Vinson

Citizenship: U.S.A.

Education:

1977	B.S.	Zoology, Duke University
1981	M.S.	Biology, University of Virginia
1987	Ph.D.	Biology, University of Virginia

Professional Experience:

1977	Duke University Marine Laboratory (student)
1980	Woods Hole Marine Biology Laboratory (student)
1987-1990	American Cancer Society Postdoctoral Fellowship, Carnegie Institution of Washington, Baltimore, MD
1991-2000	Senior Staff Fellow, Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD
2000-Present	Investigator, Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD

Teaching Experience:

1980	Teaching Assistant, Introductory Biology, University of Virginia
1981	Teaching Assistant, Introductory Biology, University of Virginia
1984	Teaching Assistant, Embryology and Anatomy, University of Virginia
1989	Instructor, Course to Isolate DNA Binding Proteins, Piscataway, NJ
1992	Instructor, EMBO Course on DNA-Protein Interactions, Naples, Italy

Awards And Honors:

1981	Masters Degree with Distinction
1982-1987	National Institutes of Health Predoctoral Fellow
1986-1987	University Fellow
1987	Andrew Fleming Award (Best thesis of the year)
1987-1990	American Cancer Society Postdoctoral Fellowship

Patent Applications:

Patents Allowed

Vinson, C. and Krylov, D.: U.S. serial No. 08/690,011, Filed July 31, 1995. Extension of a protein- protein interaction surface to inactivate the function of a cellular protein.

Patents Pending

Vinson, C. and Krylov, D.: Divisional Application U.S. serial No.: 09/299,495; Files April 26, 1999: Extension of a protein-protein interaction surface to inactivate the function of a cellular protein. filed April 1999.

Invited Lectures:

1992

Columbia University Medical School, Microbiology Department, New York, New York

LME, NICHD, Bethesda MD.

Johns Hopkins University Biology Department, Baltimore MD.

NINDS, Bethesda MD.

EMBO course instructor, Protein-DNA interactions, Naples Italy

Division of Cancer Prevention and Control, NCI, Bethesda MD.

1993

University of Maryland, Baltimore Campus, Baltimore MD.

International Business Conference: Transcription Factors, San Francisco, CA.

1994

DuPont Merck, Wilmington Delaware:

Bristol Myers Squibb, Princeton NJ

University of Southern California: Biology Department, Los Angeles, CA.

Brown University, Biology Department, Providence, RI

FAES, NIH - "Protein-DNA Interactions", Bethesda, MD

University of Arkansas, Biochemistry Department, Little Rock, AK

University of Texas at Galveston, Biochemistry Department, Galveston, TX

1995

Proceeding of the Ninth Conversation: Biomolecular Sterodynamics, Ithaca NY

NHLBI, Bethesda MD.

Stellenboch University, Biochemistry Department, Cape Town, South Africa

1996

Mount Sinai Medical School, New York, NY

FAES, NIH - "Protein-DNA Interactions", Bethesda, MD.

Ohio State University; Department of Medical Biochemistry, Columbus, OH

14th annual Mechanism of B-cell neoplasia, Bethesda MD.

1997

Liver meeting, Cold Spring Harbor Laboratories, Long Island NY

Penn State University, Chemistry Department, State College, PA

University of Virginia, Biology Department, Charlottesville, VA

University of the West Cape, Biochemistry Department, Cape Town South Africa

1998

Purdue University - "The Chemistry and Biology of Coiled Coils", West Lafayette, Indiana.
West Virginia University - "The Chemistry and Biology of Coiled Coils", Morgantown, WV

NAIMS: Building 6 - "The Chemistry and Biology of Coiled Coils", NIH, Bethesda, MD

Hood College - Experimental Therapeutics of Human Cancer, "Adenoviral Delivery of an AP-1 Dominant Negatives Makes Cis-Platinum Resistant Ovarian Cells Sensitive to Killing", Frederick, MD

University of the Witwitterstrand - "Transcriptional Control of Cell Growth", South Africa.

University of the Western Cape, South African National Bioformatics Institute (SANBI), "Transcriptional Control of Cell Growth", Belleville, South Africa.

University of Cape Town Medical School- "The Chemistry and Biology of Coiled Coils", South Africa.

Medical Research Council - "The Chemistry and Biology of Coiled Coils", South Africa

FAES, NIH – "Protein-Protein Interactions", Bethesda, MD

FAES, NIH – "Protein-DNA Interactions", Bethesda, MD

Developmental Therapeutics Program, NCI - "Adenoviral Delivery of a AP-1 Dominant Negative Makes Cis-Platinum Resistant Ovarian Cells Sensitive to Killing", Bethesda, MD

NIH Research Festival - "Protein Engineering", Bethesda, MD

Sixth Meeting of the European Working Group on Human Gene Transfer and Therapy - "Adenoviral Delivery of a AP-1 Dominant Negative Makes Cis-Platinum Resistant Ovarian Cells Sensitive to Killing", Jerusalem, Israel.

1999

Laboratory of Metabolism- "Dominant negatives to B-ZIP proteins", Bethesda MD.

2000

Keystone Symposium – "Modulating signaling with dominant negative to B-ZIP proteins". Taos New Mexico

Mt. Sinia Medical School - "The chemistry and biology of coiled coils", New York, NY

Laboratory of Molecular Biology- "Zippers", Bethesda, MD

Tumor Biology Section, NIDCD- Dominant negative to B-Zip proteins", Bethesda MD

FAES, NIH - "Protein-Protein Interactions", Bethesda, MD

FAES, NIH - "Protein-DNA Interactions", Bethesda, MD

2001

USUHS- Department of Pharmacology: "Dominant negatives to B-ZIP proteins" Bethesda Md.

University of Pennsylvania- Department of Biochemistry and Biophysics: "Leucine zipper dimerization stability and specificity", Philadelphia Penn.

University of Pennsylvania- Institute for Environmental Medicine: Chemistry and Biology of B-ZIP transcription factors", Philadelphia Penn

NIDDK- Recovery from diabetes, a new mouse expressing A-C/EBP in fat

NCI CCR LDDR- A fluorescent anisotropy assay for B-ZIP DNA binding, a high-throughput screen

2002

FAES, NIH - "Protein-DNA Interactions", Bethesda, MD

NCI Mass Spectroscopy steering committee -. "Identification of new fat derived hormones using a "fatless" mouse"

NIDCR- "Expression of a dominant negative to JUN and C/EBP produces a "fatless" mouse"

NCI- Developmental Biology Interest Group- "Design of a "promoter" microarray"

NCI- Bioinformatics Interest Group- "DNA sequences that cluster in human promoters"

NCI- Molecular Targets Steering Committee- "AP-1 as a molecular target for cancer".

2003

NIH- Chromatin Interest group: "DNA sequences that cluster in human promoters"

NIH, NCI, Laboratory of Molecular Biology- "DNA sequences that cluster in human promoters"

NIH, NIDA, Molecular Neurobiology Branch- "Inhibiting AP-1 in the mouse striatum potentiates cocaine addiction".

University of Arkansas: "Changing skin stem cell fate by RAS and AP-1"

University of Arkansas: "Design of a promoter microarray: identification DNA sequences that cluster in human promoters".

Graz University of Technology, Austria: "Ablation of adipose tissue causes diabetes: transgenic mice models".

Graz University of Technology, Austria: Design of a promoter microarray: Identification DNA sequences that cluster in human promoters

16th IGB Meeting:Molecular Biology, Genetics and Pathology of AP-1 Transcription Factors- "Transdifferentiation of skin papillomas into sebocytes by inhibiting AP-1"

Bibliography:

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3. Vinson, C. and Adler, P. Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of *Drosophila*. Nature 329: 549-551, 1987.

4. Vinson, C. and Adler, P. A minute genetic background alters the cell-autonomous phenotype of the tricorner (trc) locus. Drosophila Inform. Serv 66: 150, 1987.

5. Vinson, C.R., LaMarco, K.L., Johnson, P.F., Landschulz, W.H and McKnight, S.L. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes Dev 2: 801 806, 1988.

6. Vinson, C., Conover, S. and Adler, P. A *Drosophila* tissue polarity locus encodes a protein containing seven potential transmembrane domains. Nature 338: 263-264, 1989.

7. Vinson, C., Sigler, P.B. and McKnight, S.L. A scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science 246: 911-916, 1989.

8. Adler, P., Vinson, C., Park, W.J., Conover, S. and Klein, L. Molecular structure of frizzled, a *Drosophila* tissue polarity gene. Genetics 126: 401-416, 1989.

9. Shuman, J., Vinson, C and McKnight, S.: Evidence of changes in protease sensitivity and subunit exchange rate on DNA binding by C/EBP. Science 249: 771-774, 1990.

10. Vinson, C. and Garcia, K.C.: Molecular model for DNA recognition by the family of basic-helix-loop helix-zipper (bHLH-Zip) proteins. The New Biologist 4: 396-403, 1992.

11. Vinson, C.: Amphipathic helices in proteins that bind to DNA: The bZIP and bHLH-Zip DNA binding motifs, in The Amphipathic Helix, Richard M. Expand, Ed., CRC Press, 1992.

12. Vinson, C., Hai, T. and Boyd, S.: Dimerization specificity upon DNA binding of the leucine zipper containing bZIP motif: prediction and rational design. Genes Dev 7: 1047-1058, 1993.

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14. Thompson, K., Vinson, C. and Freire, E.: Thermodynamic characterization of the structural ability of the coiled-coil region of the bZIP transcription factor GCN4. Biochem 32: 5491-5496, 1993.

15. Krylov, D., Mikhailenko, I. and Vinson, C.: A thermodynamic scale for leucine zipper stability

and specificity: **e** and **g** interhelical interactions. EMBO J 13: 2849-2861, 1994.

16. Krylov, D., Olive, M. and Vinson, C.: Extending dimerization interfaces: the bZIP basic region can form a coiled coil. EMBO J 14: 5329-5337, 1995.

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20. Szilak, L., Moitra, J, Krylov, D. and Vinson, C.: Phosphorylation destabilizes α-helices. Nat Struct Biol 4: 112-114, 1997.

21. Olive, M, Krylov, D, Echlin, D., Taparowsky, B., Powers, C., Gardner, K. and Vinson C.: A dominant negative to AP1 that abolishes DNA binding and oncogenesis. J Biol Chem. 272: 18586-18594, 1997.

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abnormalities associated with lipoatrophy. Diabetes 51:2727-33.

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I have reviewed 106 manuscripts during my time at the NIH in the following journals: Science, PNAS, Molecular and Cellular Biology, Journal of Biological Chemistry, Nucleic Acids Research, Gene, Journal of Molecular Biology, Oncogene, EMBO Express, Molecular Cancer Research, Biotechniques, Protein Science, and FEBS Letters.

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A Dominant Negative to Activation Protein-1 (AP1) That Abolishes DNA Binding and Inhibits Oncogenesis*

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We describe a dominant negative (DN) to activation protein-1 (AP1) that inhibits DNA binding in an equimolar competition. AP1 is a heterodimer of the oncogenes Fos and Jun, members of the bZIP family of transcription factors. The DN, termed A-Fos, consists of a newly designed acidic amphipathic protein sequence appended onto the N-terminus of the Fos leucine zipper, replacing the normal basic region critical for DNA binding. The acidic extension and the Jun basic region form a heterodimeric coiled coil structure that stabilizes the complex over 3000-fold and prevents the basic region of Jun from binding to DNA. Gel shift assays indicate that A-Fos can inactivate the DNA binding of a Fos:Jun heterodimer in an equimolar competition. Transient transfection assays indicate that A-Fos inhibits Jun-dependent transactivation. Both the acidic extension and the Fos leucine zipper are critical for this inhibition. Expression of A-Fos in mouse fibroblasts inhibits focus formation more than colony formation, reflecting the ability of A-Fos to interfere with the AP1 biological functions in mammalian cells. This reagent is more potent than a deletion of either the Fos or Jun transactivation domain, which has been used previously as a dominant negative to AP1 activity.

The activation protein-1 (AP1)[1] transcription factors are immediate early response genes involved in a diverse set of transcriptional regulatory processes (1). The AP1 complex consists of a heterodimer of a Fos family member and a Jun family member. This complex binds the consensus DNA sequence (TGAGTCA) (termed AP1) sites found in a variety of promoters (2, 3). The Fos family contains four proteins (c-Fos, Fos-B, Fra-1, and Fra-2) (4–6), while the Jun family is composed of three (c-Jun, Jun-B, and Jun-D) (7–10). Fos and Jun are members of the bZIP family of sequence-specific dimeric DNA-binding proteins (11). The C-terminal half of the bZIP domain is amphipathic, containing a heptad repeat of leucines that is critical for the dimerization of bZIP proteins (12, 13). The N-terminal half of the long bipartite α-helix is the basic region that is critical for sequence-specific DNA binding (14–16).

To dissect the function of the AP1 complex in cellular processes, investigators have used dominant negatives (DNs) to AP1 consisting of a deletion of the transactivation domain of either a Jun family member (17–19) or a Fos family member (20, 21). These truncated Fos or Jun proteins dimerize with endogenous transcription factors, which results in the loss of AP1 activity (21, 22). A conceptual disadvantage with this strategy is that the heterodimer between the endogenous transcription factor and the dominant negative still binds DNA, which makes it difficult to document a change in DNA occupancy that correlates with the expression of the dominant negative. A dominant negative that is deleted for the DNA binding domain would overcome this type of problem, but such potential dominant negatives do not work well because of the stabilization that occurs when bZIP proteins bind DNA (16, 23). We are interested in developing DNs (24) that stoichiometrically inhibit the sequence-specific DNA binding of the AP1 complex. These reagents should inhibit AP1 DNA binding *in vivo*, thus allowing us to monitor occupancy of AP1 *cis* elements *in vivo* (25).

We previously demonstrated that the DNA binding of the bZIP protein C/EBP could be inhibited stoichiometrically by appending an amphipathic acidic extension to the N-terminus of the C/EBP leucine zipper (26). We explored the generality of this strategy by appending the same acidic extension onto the Fos leucine zipper. This construct (4H-Fos) was not able to inhibit AP1 DNA binding in an equimolar competition. This paper describes a newly designed amphipathic acidic extension (termed A- or N4H-), which, when appended onto the N-terminus of the Fos leucine zipper, is able to inhibit the DNA binding of AP1 in an equimolar competition. When expressed in mammalian cells, A-Fos inhibits Jun-dependent transactivation and dramatically reduces Ha-*ras*-mediated cellular transformation in a leucine zipper-dependent fashion.

EXPERIMENTAL PROCEDURES

Proteins—The Fos, Jun, VBP, and CREB bZIP domains were constructed by polymerase chain reaction and cloned into the prokaryotic expression vector pT5 as *NdeI-Hin*dIII fragments (26). All of the proteins have a 13-amino acid N-terminal Ø10 leader ASMTGGQQMGRDP.

The human Fos bZIP domain spans from Lys^{128} to Asp^{208}; chicken Jun bZIP domain spans from Ser^{222} to Phe^{310}, the natural COOH terminus; chicken VBP bZIP domain spans from Lys^{232} to Leu^{311}, the COOH terminus; mouse CREB bZIP domain spans from Leu^{274} to Asp^{341}, the COOH terminus. The C/EBP bZIP domain has been described previously (26).

The protein sequences of the acidic extensions of the dominant negatives are as follows. The last L in the following sequences is the first **d** position (see Fig. 1) of the Fos leucine zipper (14), and for cloning

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[1] The abbreviations used are: AP1, activation protein-1; DN, dominant negative; C/EBP, CAAT/enhancer-binding protein; VBP, vitellogenin-binding protein; GBF1, G-box binding factor-1; CREB, cAMP response element-binding protein; CAT, chloramphenicol acetyl transferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; CMV, cytomegalovirus; CE, colony forming efficiency; FFE, focus forming efficiency; CD, circular dichroism.

convenience, the Q following the first L of the Fos leucine zipper has been changed to an E to produce a *Xho*I site: 4H-Fos, DLEQRAEELARENEELEKEAEELEQENAE*L*E; A-Fos, DLEQRAEELARENEELEKEAEELEQE*L*AELE. *L* is the amino acid changed in the new acidic extension. The following VBP, CREB, and C/EBP leucine zippers have been cloned downstream the acidic extension as *Xho*I-*Hin*dIII fragments. The construct A-VBP contains Asp in the a position of the acidic extension. The acidic extensions fused to the leucine zippers have been cloned as *Nde*I-*Hin*dIII fragments by polymerase chain reaction into the pT5 vector. The construct 0H-Fos contains the Fos leucine zipper that spans from Leu^{165} to Asn^{208} and also has the mutation Q to E mentioned above.

Protein Purification—Proteins were expressed in *Escherichia coli*, and those capable of binding DNA were purified over a heparin column as described previously (26), and subsequently purified over a Rainin HPLC system using a C_{18} column chromatographed from 0 to 100% acetonitrile in 0.1% trifluoroacetic acid. The proteins lacking DNA binding domains were purified over a hydroxylapatite column, eluted with 200 mM phosphate, and subsequently purified on a Rainin HPLC system as just described. The protein purification protocol was modified to purify the Jun bZIP protein. The initial pellet of the Jun sample was resuspended in 1 M KCl and centrifuged at 25,000 rpm. The pellet was gently brought to 5 M urea, sonicated, heated at 65 °C for 15 min, and centrifuged, and the supernatant was isolated. The proteins were dialyzed to 50 mM KCl, 20 mM Tris, pH 8, 1 mM EDTA and loaded onto a heparin column as described before (26). The molar concentrations were calculated as described previously (26). The AP1 complex was purified from T cells as described previously (45).

Circular Dichroism—T_m values were calculated as described before (46), converted to $K_{d\,(37)}$ and $\Delta G(37)$ using a ΔC_p of −1.4 kcal/mol/ °C calculated from a T_m *versus* ΔH plot for all of the proteins used in this study. All thermal melts were reversible. The spectra were recorded in a 0.5-cm cuvette.

DNA Binding Assay—Proteins (2 μl of 5×10^{-6} M dimer) were heated for 10 min at 65 °C in the presence of 1 mM dithiothreitol and added to 20 μl of the gel shift reaction buffer (25 mM Tris (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 2.5 mM dithiothreitol, 1 mg/ml bovine serum albumin, 10% glycerol), incubated for 10 min at 25 °C, and then mixed with 8 pg of the probe (^{32}P-labeled double-stranded oligonucleotide containing the AP1 site). The binding complexes were resolved on an 8% polyacrylamide gel in 0.5% TBE buffer at room temperature. The sequence of the AP1 probe is GTCAGTCAGTGACTCAATCGGTCA. The sequence of the CREB probe is GTCAGTCAGTGACGTCAATCGGTCA. The sequence of the VBP probe is GTCAGTCAGATTACGTAATATCGGTCA. The DNA binding sites are in boldface type. The conditions used for the DNA binding assay of the AP1 complex purified from T cells have been described previously (34). The supershift analysis was performed using 3 μl of anti-JunD antibody (34) or anti-NFκB antibody (Santa Cruz Biotechnology, Inc.) added to the binding reactions for 30 min before adding the AP1 probe.

Transient Transfections—Transient transfections by calcium phosphate were carried out in HepG2 cells as described previously (47). Cells were transfected with 20 μg of DNA consisting of 10 μg of reporter, 3 μg of Jun transactivator, and 3 μg of dominant negative and salmon sperm DNA. After 2 days, cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity. CAT activities were normalized to protein concentration and represented as -fold activation over the reporter plasmid alone. The conditions for transfecting the C/EBPα transactivator and the C/EBP reporter gene were identical except that 0.3 μg of transactivator and 0.3 μg of dominant negative were transfected (26). Jurkat cells (10^7 cells) were suspended in 225 μl of RPMI complete medium containing 20% fetal calf serum at 4 °C. The addition of 8 μg of the reporter construct p10, 1 μg of Rous sarcoma virus β-galactosidase, and different concentrations of the dominant negative 4H-Fos was followed by electroporation (200 V, 1180 microfarads) (48). The cells were cultured for 2 h in complete medium and then stimulated with phytohemagglutinin (2 μg/ml) and phorbol 12-myristate 13-acetate (50 ng/ml), and the cells were harvested after 18 h. CAT assays were performed in triplicate.

Western Blot—The HepG2 cells were transfected with 10 μg of expression plasmid and 10 μg of carrier DNA. The cells were harvested in 250 mM Tris, pH 8, containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin A, 1 mM leupeptin, 1 mM pepstatin, 1 mM dithiothreitol, 0.5 mM EDTA, and 0.5 mM EGTA), the extract was freeze-thawed three times, and 30 μg of extract were run on a 16% acrylamide gel. After transfer, the membrane was probed with the FLAG M2 antibody (Eastman Kodak Co.) at a concentration of 0.5 μg/ml. The ECL kit from Amersham Corp. was used for SDS polyprotein detection.

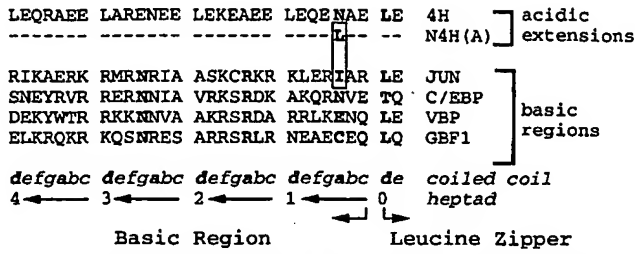

FIG. 1. **Design of an acidic extension that interacts with the Jun basic region.** The *top* of the *figure* presents the amino acid sequence of the acidic amphipathic extension (4H-) and the single amino acid change in the **a** position (Asn → Leu) needed to create the potent new acidic extension (N4H- or **A**) described in this paper. Below is the amino acid sequences of four basic regions, Jun, C/EBP, VBP, and GBF1. The *box* encloses the leucine from the new acidic extension (N4H- or A) and the isoleucine from the Jun basic region, which are thought to interact. In boldface type are the first leucine position of the zipper, the invariant asparagine and arginine of the basic region, and the **a** position amino acid, which is critical for the efficacy of the new acidic extension. The coiled coil nomenclature of the basic region extending from the leucine zipper is indicated *below* with the hydrophobic **a** and **d** positions in boldface type. The numbering of the heptads in the acidic extension is indicated.

Indirect Immunofluorescence—HepG2 cells were cultured in 1-ml slide flasks (Nunc). Cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature followed by methanol for 10 min. After blocking with 3% bovine serum albumin in PBS and 0.1% Tween 20 for 30 min at room temperature, slides were incubated with a 1:200 FLAG M2 antibody and 3% bovine serum albumin in PBS for 2 h, followed by incubation for 1 h with fluorescein isothiocyanate-conjugated rat anti-mouse antibody used at a 1:200 dilution in PBS containing 3% bovine serum albumin. After each incubation with antibodies, cells were extensively washed with PBS, 0.05% Tween 20 twice for 10 min each at room temperature.

Eukaryotic Plasmids—The eukaryotic expression plasmid containing chicken Jun is driven by the CMV promoter and has been described elsewhere (21). The CAT reporter plasmid containing a single AP1 binding site has been constructed by inserting the AP1 consensus site AGCTTGGATCCAGATCGAGCCCCAATGACTCATCATAGA in front of a minimal promoter p-35 Alb CAT described previously (47) and has been used for transfection in HepG2 cells. The CAT reporter plasmid p10 used for transfection experiments in Jurkat cells consists of a chimeric c-*fos* promoter gene fusion carrying the Gibbon ape leukemia virus-TPA-responsive element enhancer and has been described previously (49). Dominant negative coding sequences (0H-Fos, 4H-Fos, A-Fos, A-VBP, 4H-CREB, A-CREB) were cloned as *Nde*I-*Hin*dIII fragments into pRc/CMV vector (Invitrogen) modified to contain a N-terminal hemagglutinin epitope (MYPYDVPDYA) pRc/CMV566 or an N-terminal FLAG epitope (MDYKDDDK) and a new polylinker. The *Nde*I-*Hin*dIII fragments were obtained from the prokaryotic expression vector pT5 in which the dominant negatives had been cloned previously (see "Proteins").

Stable Transfections—Stable transfection of the murine fibroblast cell line $C_3H10T1/2$ (ATCC number CCL226) was performed as described previously (29) using the calcium phosphate DNA precipitation method. Individual precipitates containing 200 ng of pT24 Ha-*ras* (30) and 600 ng of each pRc/CMV566 construct were added to two 100-mm tissue culture dishes, each seeded 24 h prior to transfection with 5×10^5 cells and treated 2 h before the addition of precipitates with medium containing 10 μg/ml chloroquine (Sigma). To assess focus formation, 24 h after transfection, the cultures were split 1:3 and maintained in basal modified Eagle's medium supplemented with 5% fetal calf serum. To assess colony formation, ⅙ of the cell suspension obtained from both plates was further divided 1:6 and maintained in basal modified Eagle's medium supplemented with 10% fetal calf serum and 400 μg/ml Geneticin (G-418 sulfate, Life Technologies, Inc.). After 14 days, the plates were stained with Giemsa (EM Diagnostic Systems, Darmstadt, Germany), and the efficiency of both focus formation and colony formation was determined by visual inspection. The efficiency of focus formation was calculated for each experimental group based on the number of foci obtained from a parallel group transfected with either Ha-*ras* or with Ha-*ras* plus pRc/CMV566 DNA, which is set at 1.00. The efficiency of colony formation was calculated for each group based on the number of

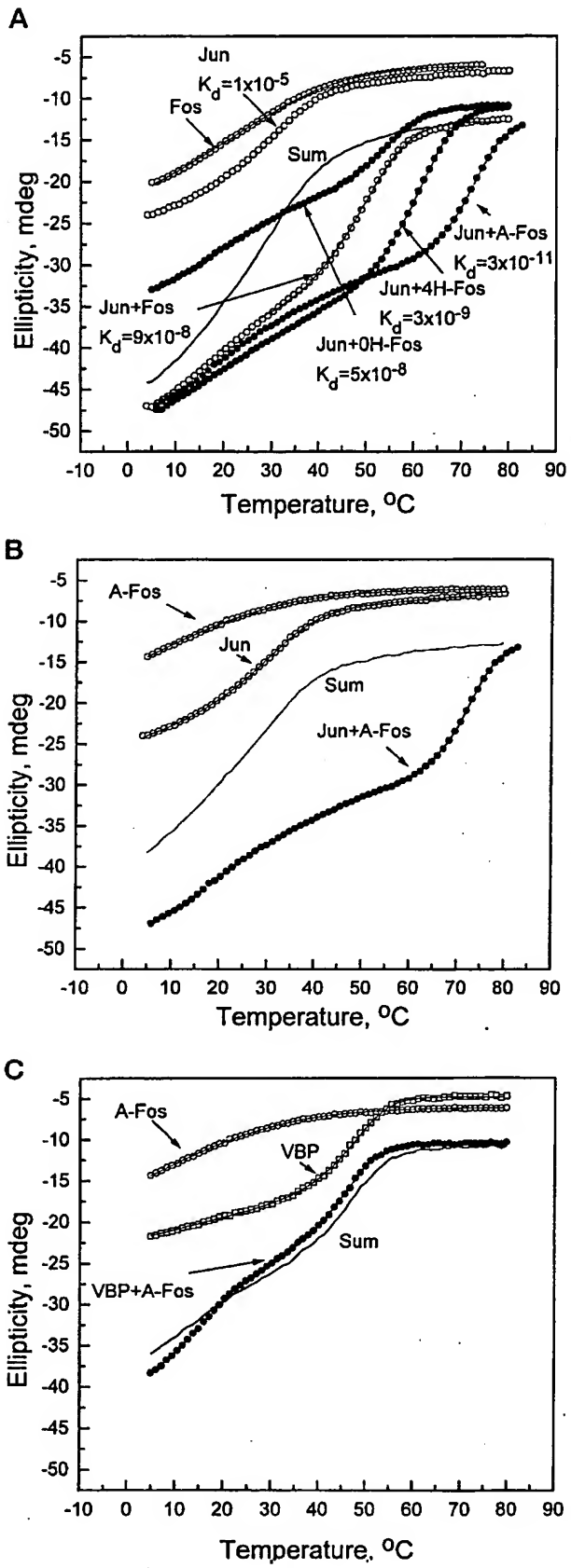

FIG. 2. **Stability of the acidic extension appended to Fos zipper with Jun.** *A*, CD thermal melting curves at 222 nm of 1) Jun, 2) Fos, 3) Jun + Fos, 4) Jun + 0H-Fos (the Fos zipper without the basic region), 5) Jun + 4H-Fos, and 6) Jun + A-Fos. The melts of Fos, Jun, and the mixture (Jun + Fos) are shown with *open circles*. The *solid line* labeled *Sum* is what we would expect if Fos and Jun did not interact. The melts of the mixtures of Jun with the three potential dominant negatives (Jun + 0H-Fos, Jun + 4H-Fos, and Jun + A-Fos) are shown with closed points. The fitted curve through each of the data sets was used to calculate T_m as described previously. The $K_{d(37)}$ for each mixture are shown in M (26). *B*, CD thermal melting curves at 222 nm of 1) A-Fos, 2) Jun, and 3) Jun + A-Fos. The *solid line* labeled *Sum* is what we would expect if Jun and A-Fos did not interact. *C*, CD thermal melting curves at 222 nm of 1) A-Fos, 2) VBP, and 3) VBP+A-Fos. The *solid line* labeled *Sum* is what we would expect if VBP and A-Fos did not interact.

colonies obtained from a group transfected with Ha-*ras* plus pRc/CMV566, which is set at 1.00. The values reported (in most instances) have been calculated from the numbers obtained from multiple, independent experiments. The ratio of colony forming efficiency (CE) to focus formation efficiency (FFE) assesses the relative contribution of a decrease in CE to the observed level of FFE. CE/FFE values greater than 1.0 are considered significant.

RESULTS

Design of Acidic Amphipathic Extension That Forms a Coiled Coil with the Jun Basic Region—Previously, we showed that three bZIP basic regions from C/EBP, VBP, and GBF1, when appended onto the N-terminus of the C/EBP leucine zipper, were able to form a heterodimeric coiled coil structure with a designed acidic amphipathic protein sequence. The acidic amphipathic extension had been appended onto the N-terminus of a leucine zipper designed to preferentially interact with the C/EBP leucine zipper (Fig. 1) (26, 27). This acid amphipathic extension (4H-) of the leucine zipper created a potent DN that heterodimerized with the bZIP protein C/EBP and prevented DNA binding. We explored the generality of this method by appending the acidic amphipathic extension onto the Fos leucine zipper in an attempt to inhibit Fos:Jun DNA binding. The rationale was that the acidic extension would electrostatically mimic DNA and provide the Jun basic region with an alternate interaction surface. The Jun basic region, instead of binding in the major groove of DNA, would form a heterodimeric coiled coil structure with the acidic amphipathic protein sequence.

The above strategy was unsuccessful because the Jun basic region is different from the three basic regions examined previously (Fig. 1). There is a hydrophobic amino acid (isoleucine) in the **a** position immediately N-terminal of the first **d** position of the Jun leucine zipper, while the three previous basic regions (C/EBP, VBP, and GBF1) contained a polar amino acid (asparagine, glutamate, or cysteine) in this position. Previously, we placed an asparagine in the corresponding position of the acidic extension (4H-) to create a polar interaction in the hydrophobic interface as is seen in the leucine zipper, which also contains an N in the **a** position (28). In the new acidic extension (N4H-) (Fig. 1), we have replaced this polar asparagine within 4H- with a hydrophobic leucine, reasoning that the hydrophobic isoleucine of the Jun basic region would interact more favorably with a leucine than an asparagine. For simplicity, we refer to N4H-Fos as A-Fos, where A refers to acidic extension.

The New Acidic Extension of the Fos Zipper Stabilizes the Interaction with the Jun bZIP Domain 3000-fold—The thermal stability of mixtures of the Jun and Fos bZIP domains and the Jun bZIP domain with different potential dominant negatives was monitored using CD spectroscopy (Fig. 2, Table I). The dissociation constants were calculated at 37 °C because they provide information about dimerization *in vivo*. The Fos bZIP domain is so unstable that we were unable to determine reliably a dissociation constant. The Jun bZIP domain, however, does produce an interpretable thermal melt with a $K_{d\,(37)} = 10^{-5}$ M (Table I). The mixture of Fos and Jun forms a heterodimer. This is demonstrated by the greater stability of the

TABLE I

Thermal stability of Fos, Jun, Fos + Jun, and Jun with three potential dominant negatives

Presented below are the melting temperature (T_m, °C), ΔG, and K_d at 37 °C or 25 °C for CD thermal melts of a variety of proteins either as homodimers or an equimolar mixture of two proteins. If the mixture has a higher melting temperature than either homodimer alone, we infer that the mixture sample is composed of heterodimers. The root mean square error in ΔG is 0.2 kcal/mol and 0.5 kcal/mol for homo- and heterodimers, respectively.

Protein	Homodimer $T_m(\Delta G)K_{d\,(37)}$	Heterodimer with Jun $T_m(\Delta G)K_{d\,(37)}$	Heterodimer with Jun $T_m(\Delta G)K_{d\,(25)}$	Heterodimer with C/EBP $T_m(\Delta G)K_{d\,(37)}$
Jun	29.9 (−7.1)1*e* − 5			
Fos	25.0[a]	49.9 (−10.0)9*e* − 8	49.9 (−10.8)1.3*e* − 8	
0H-Fos		53.4 (−10.4)5*e* − 8	53.4 (−11)8.5*e* − 8	
4H-Fos	25.1 (−7.0)1*e* − 5	61.1 (−12.2)3*e* − 9	61.1 (−12.9)4.2*e* − 10	
A-Fos		72.1 (−15.0)3*e* − 11	72.1 (−15.8)2.8*e* − 12	
C/EBP	49.5 (−9.7)2*e* − 7			
4H-C/EBP	54.3 (−11.3)1*e* − 8			63.8 (−13.0)7*e* − 10
A-C/EBP	62.7 (−12.1)4*e* − 9			66.7 (−13.1)8*e* − 10

[a] The Fos bZIP domain is so unstable that a reliable K_d could not be measured.

mixture ($K_{d\,(37)} = 9 \times 10^{-8}$ M), which is greater than the sum of the individual Fos and Jun thermal melts (*solid line*) (Fig. 2*A*). The deduced $K_{d\,(25)}$ for a Fos and Jun mixture is 1.3×10^{-8} M, which is similar to the value of $K_{d\,(25)} = 2.3 \times 10^{-8}$ M reported earlier using fluorescence energy transfer assay (23).

We then examined the thermal stability of the Jun bZIP domain mixed with three potential dominant negatives: the Fos leucine zipper without the basic region or the Fos leucine zipper with one of two different acidic extensions appended onto the N-terminus (Fig. 2*A*, Table I). The mixture of Jun with Fos without a basic region (Jun + 0H-Fos) is twice as stable ($\Delta\Delta G = -0.4$ kcal/mol) as the Jun + Fos mixture, indicating that the basic regions are repulsive; a similar result was seen with the C/EBP basic region (26). A surprising result was that the ellipticity at 6 °C for the Fos + Jun mixture was greater than for the mixture of Fos without the basic region (0H-Fos) and Jun. This suggests that the basic regions are helical in the absence of DNA, an observation that is not seen for C/EBP (26). The addition of the acidic amphipathic extension to the N-terminus of the Fos leucine zipper dramatically stabilizes the interaction with Jun. Using the previously described acidic extension (4H-Fos), we observe a 30-fold increase ($\Delta\Delta G = -1.8$ kcal/mol) in the stability of the Jun bZIP domain. The new acidic extension containing the single Asn → Leu change (A-Fos) is 3000-fold more stable ($\Delta\Delta G = -4.6$ kcal/mol) than the Jun + 0H-Fos mixture (Fig. 2, *A* and *B*, Table I). The single amino acid change increased the heterodimer stability 2.8 kcal/mol.

The specificity of the interaction of A-Fos with additional bZIP domains was determined by CD thermal denaturation. The VBP bZIP domain and A-Fos were thermally denatured, either alone or together, and no interaction was observed (Fig. 2*C*). Similar results were obtained for the mixture of 4H-Fos and C/EBP (data not shown). This suggests that the acidic extension is only able to interact with the basic region if the leucine zippers themselves are physically interacting.

To determine if the new acidic extension containing the Asn → Leu change (A-) stabilized other bZIP basic regions or was specific for a basic region containing a hydrophobic residue in the a position, we appended the new acidic sequence onto the C/EBP leucine zipper (A-C/EBP) and determined the thermal stability of mixtures with C/EBP (Table I). C/EBP interacts similarly with both acidic extensions with the following dissociation constants: $K_{d\,(37)} = 7 \times 10^{-10}$ M for 4H-C/EBP and 8×10^{-10} M for A-C/EBP. In the context of the C/EBP leucine zipper, the new acidic extension only contributes to a 0.1 kcal/mol increase in stability, which is negligible. These data demonstrate that the Asn → Leu change produces a new acidic extension that interacts well with all bZIP basic regions examined but prefers to interact with basic regions containing a hydrophobic residue in the a position, *e.g.* the Jun basic region.

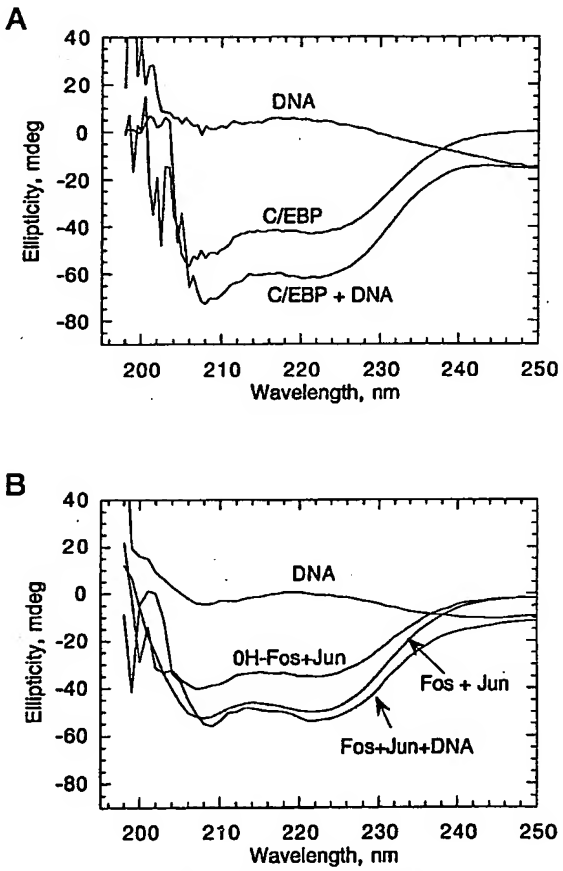

FIG. 3. **Structure of C/EBP, Jun, and Fos basic domains with or without DNA.** *A*, CD spectra from 200 to 250 nm of 1) 25-base pair double-stranded DNA containing the C/EBP site, 2) C/EBP bZIP domain, and 3) C/EBP + DNA. *B*, CD spectra containing 1) 24-base pair double-stranded DNA containing the AP1 site, 2) Fos + Jun heterodimers, 3) 0H-Fos + Jun, and 4) Fos + Jun + DNA in 10 mM phosphate, pH 7.4, 150 mM KCl, 0.25 mM EDTA. The concentration of all samples was at 2 μM dimer and 2 μM DNA. All of the spectra were measured at 6 °C.

The Acidic Extension Does Not Increase the α-Helical Content of the Jun Basic Domain—A puzzling result from the thermal denaturation experiments was the amount of ellipticity at 222 nm indicative of α-helical structure seen at low temperatures when the samples are dimeric. The mixture of the Jun bZIP

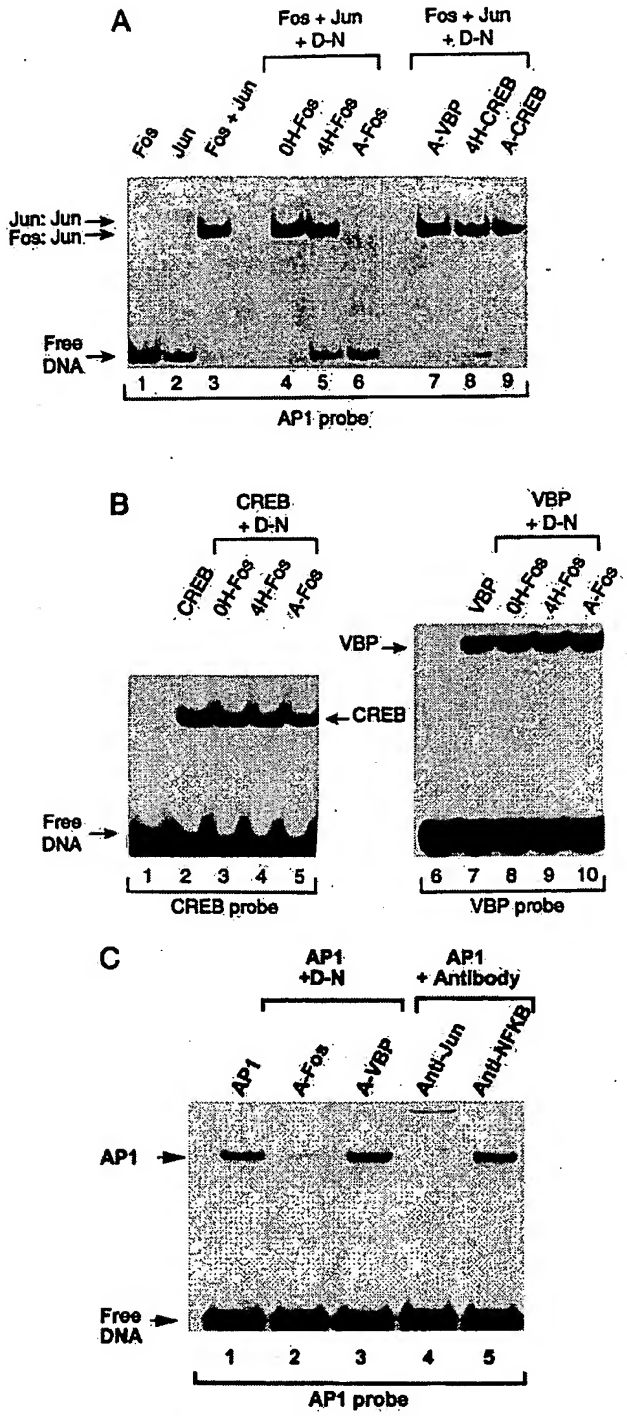

FIG. 4. **Acidic extension appended to the Fos zipper inhibits Fos:Jun DNA binding.** *A*, the *left panel* shows a gel retardation assay of Fos (*lane 1*), Jun (*lane 2*), and Fos + Jun (*lane 3*), binding to an AP1 (TGAGTCA) specific DNA probe that is 24 base pairs long. Inhibition of Fos:Jun DNA binding was measured following the addition of a 1.1 molar equivalent of different DNs to the Fos:Jun complex. *Lane 4*, Fos + Jun + 0H-Fos; *lane 5*, Fos + Jun + 4H-Fos; *lane 6*, Fos + Jun + A-Fos. A-Fos is able to inhibit Fos:Jun DNA binding activity. The inhibition of Fos:Jun DNA binding is leucine zipper-specific. The DNA binding of Fos:Jun was challenged with different proteins containing the acidic extension appended to different leucine zippers. A 1.1 molar equivalent of A-VBP (*lane 7*), 4H-CREB (*lane 8*), and A-CREB (*lane 9*) was added to Fos + Jun and did not inhibit Fos:Jun DNA binding. *B*, A-Fos does not inhibit the sequence-specific DNA binding properties of CREB or VBP. Adding a 1.1 molar equivalent of 0H-Fos (*lanes 3* and *8*), 4H-Fos (*lanes 4* and *9*), or A-Fos (*lanes 5* and *10*) does not inhibit the binding of CREB or VBP to its target site DNA (*lanes 2* and 7). *C*, A-Fos inhibits the DNA binding of AP1 purified complex isolated from T cells. *Lane 1* shows the DNA binding of the AP1 purified complex on a AP1 DNA probe. Adding A-Fos to the AP1 complex (*lane 2*) inhibits AP1 binding, but adding A-VBP (*lane 3*) did not inhibit AP1 DNA binding. Supershift analysis using antibodies against Jun (*lane 4*) or against NF-κB (*lane 5*) added to the binding reactions shows that this complex contains a Jun family member.

and Fos bZIP domains (Jun + Fos) has more helicity than the mixture of Jun bZIP and the Fos leucine zipper (Jun + 0H-Fos) (Fig. 2*A*). This suggests that the basic regions of Fos and Jun are helical in the absence of DNA. This result was not observed when similar experiments were done with C/EBP. The basic regions of the bZIP proteins GCN4 and C/EBP have been shown to be nonhelical in the absence of DNA and to become helical when bound to DNA (15, 16, 31). We measured the CD spectra from 200 to 250 nm of C/EBP and Fos:Jun in the absence and presence of sequence-specific DNA (Fig. 3). CD ellipticity at 222 nm is indicative of helicity. As reported earlier, we observe that the C/EBP bZIP domain shows a 44% increase in helicity with the addition of DNA (Fig. 3*A*) (31). In contrast, the Fos:Jun heterodimer shows only a modest 10% increase in helicity after binding sequence-specific DNA (Fig. 3*B*), an increase identical to that reported earlier (32). Interestingly, the mixture of Jun with a Fos zipper lacking the basic region (Jun + 0H-Fos) contains 40% less helicity than the mixture of the Fos and Jun bZIP domains (Fig. 3*B*). This suggests that the Jun and Fos basic regions are largely helical in the absence of DNA, unlike the C/EBP basic region, although the basic regions are repulsive.

Inhibition of AP1 DNA Binding—Gel shift experiments were undertaken to examine the number of molar equivalents of A-Fos that would be needed to inhibit the DNA binding of a mixture of Fos and Jun. Published data indicate that a Fos:Jun heterodimer binds DNA with a $K_{d\,(25)} = 2 \times 10^{-10}$ M (33). CD experiments presented in Table I indicate that A-Fos heterodimerizes with Jun with a $K_{d\,(25)} = 2.8 \times 10^{-12}$ M. Therefore, an equimolar mixture of Fos, Jun, and A-Fos should prevent Fos:Jun heterodimers from binding DNA because of the preferred formation of the Jun:A-Fos heterodimer. Fos, Jun, or a Fos + Jun mixture was incubated with a labeled 24-base pair oligonucleotide containing a single AP1 site and tested for DNA binding using a gel shift assay. Fig. 4*A* shows that Fos does not bind AP1 DNA (*lane 1*) but that Jun:Jun homodimers bind slightly (*lane 2*) and Fos:Jun heterodimers bind well (*lane 3*). One molar equivalent of A-Fos is able to totally inhibit Fos:Jun DNA binding (*lane 6*). The Fos leucine zipper without the acidic extension (0H-Fos) at equimolar conditions does not inhibit Fos:Jun binding (*lane 4*), while the previously described acidic extension (4H-Fos) inhibits Fos:Jun binding only partially as indicated by the amount of free probe remaining in the reaction. These data demonstrate that the mutation Asn → Leu has a dramatic effect on the ability of a DN to prevent AP1 DNA binding. At equimolar concentrations, 0H-Fos and 4H-Fos are not expected to inhibit Fos:Jun DNA binding, because they heterodimerize with Jun with a lower affinity than Fos:Jun bound to DNA. Indeed, Jun:0H-Fos shows a $K_{d\,(25)} = 8.5 \times 10^{-9}$ M and Jun:4H-Fos shows a $K_{d\,(25)} = 4.2 \times 10^{-10}$ M.

To examine the specificity of the A-Fos ability to inhibit a Fos:Jun mixture from binding DNA, we undertook two controls. The first control was used to determine whether the leucine zippers of 4H-Fos and A-Fos are critical for the inhibition of Fos:Jun DNA binding. Chimeric proteins were generated where the Fos leucine zipper was replaced with the VBP or the CREB leucine zipper. Fos:Jun DNA binding is not inhibited

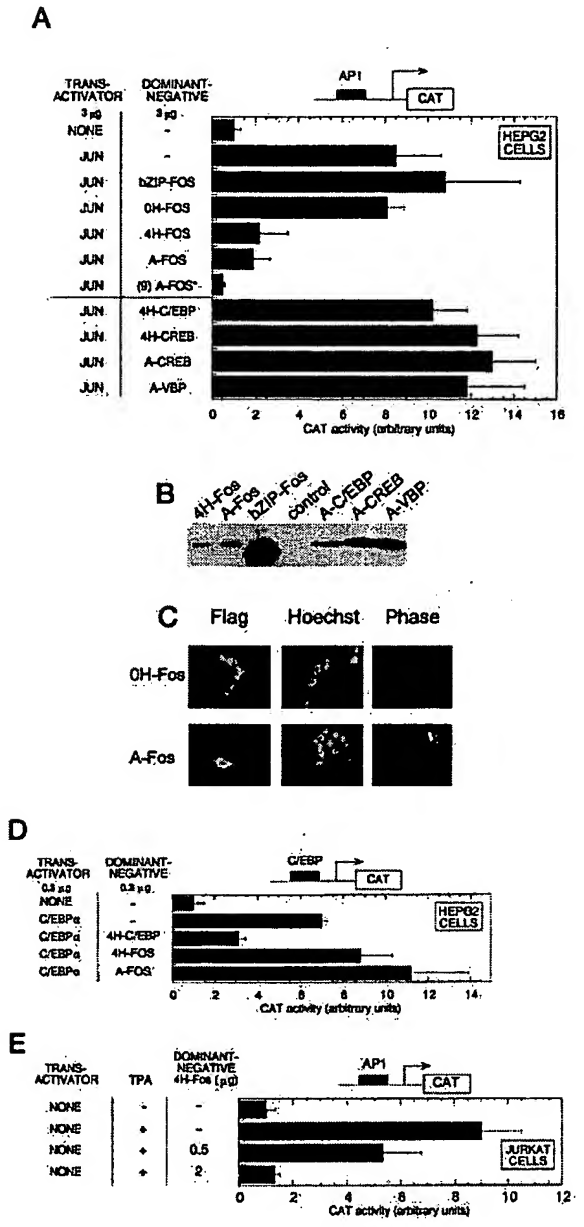

FIG. 5. **Acidic extension to the Fos zipper inhibits Jun transactivation.** *A*, human hepatoma cells (HepG2) were transiently transfected with three plasmids, a CAT expression plasmid driven by a single AP1 *cis* element, the Jun transactivator, and different dominant negatives. CAT activity was measured and expressed as -fold activation ± S.D. relative to the activity of the reporter plasmid alone. Jun transactivates a single AP1 site approximately 8-fold. The Fos bZIP domain and the Fos leucine zipper are not effective at inhibiting Jun-dependent transactivation. The inhibition is leucine zipper-dependent. Replacing the Fos zipper with the CREB, VBP, or C/EBP zippers does not affect Jun transactivation. Transfections were carried out using 10 μg of the reporter containing a single AP1 *cis* element, 3 μg of CMV Jun and 3 μg of the different dominant negatives, except A-Fos labeled with a *star* for which 9 μg were also transfected. *B*, Western blot analyses of whole-cell extract prepared from HepG2 nontransfected cells (control) and cells transfected with different DNs. The blot was incubated with an anti-FLAG antibody. *C*, indirect immunofluorescence staining of FLAG epitope-tagged proteins 0H-Fos and A-Fos. HepG2 cells were transfected with 0H-Fos and A-Fos, fixed, and stained with FLAG antiserum (*left*), Hoescht 33342 (*center*), or viewed through phase (*right*). *D*, 4H-Fos and A-Fos do not inhibit C/EBP transactivation properties. Transfections were carried out in HepG2 cells as described in *A*, except that the reporter gene contains a single C/EBP *cis* element, and the transactivator is C/EBPα. C/EBPα is able to transactivate the C/EBP-containing promoter and is not inhibited by 4H-Fos or A-Fos. Transactivations were carried out using 10 μg of the reporter containing the C/EBP *cis* element, 0.3 μg of mouse sarcoma virus C/EBPα, and 0.3 μg of 4H-Fos or 0.3 μg of A-Fos. *E*, 4H-Fos inhibits TPA-induced T cell activation. Jurkat cells were transfected with 8 μg of the reporter plasmid p10 and either 0.5 or 2 μg of 4H-Fos expression vector and induced with phorbol 12-myristate 13-acetate and phytohemagglutinin for 18 h. CAT activity was measured and expressed as -fold activation ± S.D. relative to the activity of the reporter plasmid alone (noninduced).

by A-VBP (*lane 7*), by 4H-CREB (*lane 8*), or by A-CREB (*lane 9*), indicating that the inhibition of Fos:Jun DNA binding is dependent on the Fos leucine zipper. The second control asked if A-Fos inhibits the binding of other bZIP proteins to DNA. To test this, we incubated equal molar quantities of the Fos DNs with two bZIP proteins, CREB or VBP. In Fig. 4*B*, we observe that 0H-Fos (*lane 3*), 4H-Fos (*lane 4*), and A-Fos (*lane 5*) do not inhibit the sequence-specific DNA binding of CREB (*lane 2*). The same results are obtained for sequence-specific VBP binding (Fig. 4*B*, *lanes 8–10* and *7*).

The ability of A-Fos to inhibit the DNA binding of a native AP1 complex was determined (Fig. 4*C*). Purified AP1 protein complex, isolated from T cells, was bound to an AP1-specific DNA sequence (34). A-Fos was able to totally inhibit DNA binding (*lane 2*), while the same acidic extension appended to the VBP leucine zipper was not able to inhibit DNA binding (*lane 3*). The composition of the AP1 purified complex was examined by performing an AP1 DNA binding assay in the presence of supershifting antibodies directed against Jun family proteins (*lane 4*). The AP1 complex is supershifted by a Jun but not an NF-κB antibody. These results demonstrate two points: A-Fos is able to inhibit the DNA binding of a native AP1 complex, and the inhibition of DNA binding caused by A-Fos is leucine zipper-dependent (Fig. 3*C*, *lanes 3* and *4*).

Inhibition of AP1 Transactivation—A transient transfection assay in a human hepatoma cell line (HepG2) was employed to examine the dominant negative properties of A-Fos. HepG2 cells were co-transfected with the Jun transactivator and a CAT reporter gene containing a single AP1 binding site. Jun is able to transactivate this promoter 10-fold (Fig. 5*A*). Four different potential DNs were tested for their ability to inhibit Jun transactivation at a 1:1 molar ratio, an experimental condition where we tried to avoid overexpression of the DN. These DNs are the Fos bZIP domain with the transactivation domain deleted (bZIP-Fos), the Fos leucine zipper (0H-Fos), and the Fos leucine zipper with the two acidic amphipathic extensions (4H- and A-) appended onto the N-terminus. Neither the Fos bZIP domain nor the Fos leucine zipper, two possible DNs that could occur by the simple deletion within the *fos* gene, were able to inhibit transactivation under the experimental conditions used. 4H-Fos and A-Fos inhibited Jun transactivation over 80%. Complete inhibition is observed when a 3:1 molar ratio of the A-Fos to Jun transactivator is used. The expression of the different DN proteins was checked by Western blot (Fig. 5*B*) using the N-terminal FLAG epitope present on each of the DNs. bZIP-Fos was overexpressed in HepG2 cells compared with A-Fos, but it is not an efficient inhibitor of Jun transactivation. 0H-Fos can hardly be detected on Western blot (data not shown), but they can be seen at the same density as A-Fos using an immunofluorescence assay (Fig. 5*C*). We think the 0H-Fos epitope is proteolysed during the cell extract preparation, a problem we have encountered previously (26).

To investigate whether A-Fos inhibition of Jun transactivation is dependent on the Fos leucine zipper (Fig. 5*A*), chimeric DNs were generated where the Fos leucine zipper was replaced by three different leucine zippers (C/EBP, CREB, or VBP). These chimeric DNs are not able to inhibit Jun transactivation, indicating that A-Fos is acting in a leucine zipper-specific

TABLE II

Inhibition of cellular growth and transformation by dominant negatives to AP1

FFE is expressed for each group relative to the number of foci obtained in the control group co-transfected with Ha-*ras* and the empty vector pRc/CMV566, which is set at 1.00. CE is expressed for each group relative to the number of colonies obtained in the control group co-transfected with Ha-*ras* and the empty vector pRc/CMV566, which is set at 1.00. CE/FFE corresponds to the ratio of CE to FFE. The concentrations of plasmids used in these experiments are described under "Experimental Procedures."

	Number of foci				FFE	Number of G418^r colonies		CE	CE/FFE
	Exp. 1[a]	Exp. 2	Exp. 3	Exp. 4		Exp. 1	Exp. 2		
Ha-*ras*	207	134	123	156	1.08				
Ha-*ras* + vector		152		135	1.00	427	471	1.00	1.00
Ha-*ras* + bZIP-Fos			86	80	0.59		349	0.78	1.32
Ha-*ras* + 0H-Fos	75	54		42	0.40	288	233	0.58	1.45
Ha-*ras* + A-Fos	53	41		35	0.30	314	249	0.63	2.10
Ha-*ras*	134				1.00				
Ha-*ras* + 4H-CREB	97				0.72				
Ha-*ras* + 4H-C/EBP	161				1.20				

[a] Exp., experiment.

manner.

To examine whether A-Fos is acting nonspecifically by inactivating transcription, we determined whether A-Fos inhibited the transactivation of C/EBPα, another bZIP transactivator. HepG2 cells were transfected with a CAT reporter gene containing 1) a single C/EBP binding site in the promoter and C/EBPα alone, 2) C/EBPα plus 4H-Fos, or 3) C/EBPα plus A-Fos (Fig. 5*D*). C/EBPα is able to activate a single C/EBP *cis* element 7-fold. Either acidic extension appended onto the N-terminus of the Fos zipper does not inhibit C/EBPα transactivation, demonstrating that the dominant negative to AP1 does not inhibit the function of other bZIP proteins.

The human Jurkat T cell line was used as a model to examine the effect of the Fos dominant negative (4H-Fos) on TPA-induced T cell activation (Fig. 5*E*). Incubation of Jurkat cells with phytohemagglutinin and phorbol 12-myristate 13-acetate phorbol ester (equivalent to TPA) results in the production of interleukin-2. The interleukin-2 promoter contains a TPA-responsive element that binds AP1 proteins and is a major target for the phorbol ester response. Jurkat cells were transfected with a CAT reporter gene containing three TPA-responsive elements. The addition of TPA results in a 10-fold activation of the reporter gene, and the co-transfection of 4H-Fos inhibits the TPA activation in a dose-dependent fashion. This result demonstrates that 4H-Fos is able to inhibit the transcriptional activity of a native AP1 complex in T cells.

DN to AP1 Inhibits Ha-ras-mediated Cellular Transformation More than Cell Growth—AP1 is an immediate early protein complex that plays an important role in the initiation of cellular growth (3). The activity of AP1 also is a critical downstream mediator of the proliferative effects of several oncoproteins, most notably members of the Ras family (35–37). To investigate the possibility that DNs to AP1 could be used to dissect further the role of AP1 in cellular growth and transformation, C_1H10T1/2 mouse fibroblasts were stably transfected with the human Ha-*ras* oncogene and a 3-fold molar excess of vector DNA, bZIP-Fos, 0H-Fos, or A-Fos. The transfected cells were plated as described under "Experimental Procedures" to assay for focus formation as well as to assay for the ability of DN expressing cells to produce stable, G418^r colonies (Table II). In most cases, the results from multiple, independent experiments were averaged, and the focus forming and colony forming efficiencies are expressed relative to the appropriate Ha-*ras* control. The results show that all three DN constructs inhibit both C_1H10T1/2 cell growth and Ha-*ras*-mediated cellular transformation. The Fos bZIP domain lacking a transactivation domain, the type of DN previously used (20), reduced foci formation to 59% of the Ha-*ras* control while only inhibiting colony formation to 78% of controls. Using the same concentration of input DNA, 0H-Fos inhibits foci formation to 40%, while the acidic extension (A-Fos) decreases foci formation to 30% and inhibits colony formation to only 63%. Interestingly, when the average reduction in CE is expressed relative to the average inhibition in FFE, the A-Fos construct was shown to inhibit transformation 2-fold over the level anticipated from the observed impact of this protein on C_1H10T1/2 cell growth. Reductions in focus formation similar to those seen with the DNs to AP1 were not observed when 4H-CREB and 4H-C/EBP were tested in Ha-*ras* transformation assays, indicating that the inhibition in foci formation is dependent on the Fos leucine zipper (Table II). We take these results to mean that A-Fos has the potential to be used as a highly specific DN to experimentally separate the various biological activities of AP1 in mammalian cells.

DISCUSSION

We have generated a DN protein termed A-Fos that inhibits the DNA binding of AP1 in an equimolar competition. AP1 is a heterodimer composed of two bZIP proteins, a Fos family member and a Jun family member. A-Fos contains an acidic amphipathic protein sequence appended onto the N-terminus of the Fos leucine zipper, and the acidic extension physically interacts with the Jun basic region. The interaction of the Jun basic region with the acidic extension extends the leucine zipper dimerization interface into the basic region, thus preventing the basic region in this heterodimeric structure from binding DNA (Fig. 6). The acidic protein sequence stabilizes the interaction of the Fos zipper with the Jun bZIP domain 4.6 kcal/mol or 3000-fold. This increase in heterodimer stability makes this DN an effective competitor in a stoichiometric competition. Gel shift experiments indicate that A-Fos is indeed able to inhibit Fos:Jun DNA binding in an equimolar competition experiment. Neither the Fos leucine zipper alone nor a previously described acidic extension (4H-) appended onto the N-terminus of the Fos zipper was able to inhibit Fos:Jun DNA binding, demonstrating that the newly described acidic extension is critical for the function of the A-Fos DN. The specificity of the inhibition of DNA binding was assayed by replacing the Fos leucine zipper with sequences obtained from two heterologous zipper proteins, C/EBP and VBP. These proteins were not able to inhibit Fos:Jun DNA binding, indicating that the specificity of the DN is derived from the leucine zipper and the stability of the complex from the acidic extension.

The ability of this newly designed DN (A-Fos) to inhibit the biological function of AP1 was tested in a variety of assays. A co-transfection assay using human hepatoma cells (HepG2) shows that A-Fos is able to inhibit Jun-dependent transactivation of a promoter containing a single AP1 *cis* element. When

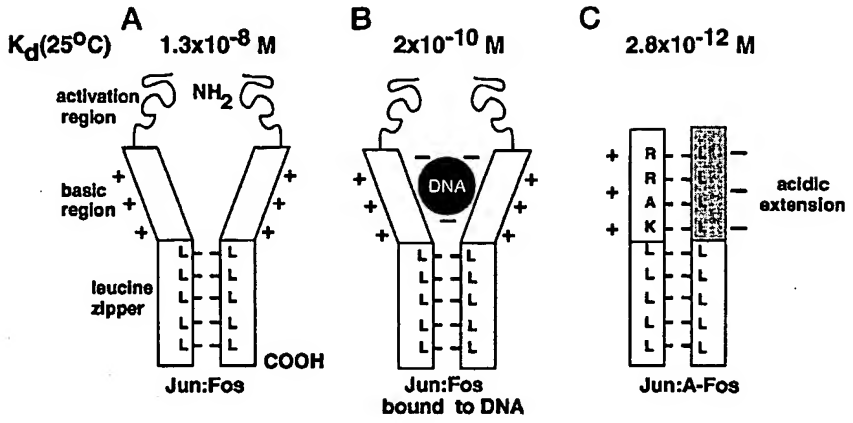

FIG. 6. **Schematic of an AP1 dominant negative.** *A*, a Jun:Fos heterodimer. *B*, Jun:Fos binding to DNA (33). *C*, Jun:A-Fos heterodimerization.

equal molar quantities of A-Fos plasmid DNA were added to the transfection, Jun-dependent transactivation was decreased to 20% of controls. When a 3-fold molar excess of DN was added, Jun-dependent transactivation was totally abolished. The specificity of the inhibition was tested using two separate approaches. The inhibition of Jun-dependent transactivation was tested by appending the acidic extension onto several other leucine zipper sequences that do not dimerize with either the Fos or Jun leucine zippers. These potential DNs did not inhibit Jun-dependent transactivation. The second approach was to test whether A-Fos inhibits transactivation by the bZIP protein C/EBPα. The transactivation of C/EBPα is not inhibited by the A-Fos DN, demonstrating that A-Fos only inhibits leucine zippers with which it can physically interact.

The human Jurkat T cell line was used as a model to examine the effect of the Fos dominant negative on TPA-induced T cell activation. Incubation of Jurkat cells with phytohemagglutinin and TPA results in the production of interleukin-2. We showed that 4H-Fos is able to inhibit the transcriptional activity of a native AP1 complex in T cells, implying that it can be used as a reagent to study the the cascade of events leading to the the activation of the interleukin-2 gene.

A cellular transformation assay in C_1H10T1/2 cells was utilized to explore whether A-Fos could inhibit Ras-dependent transformation. As an immediate early transcription complex, AP1 has been shown to be important for the initiation of cell growth (1, 38). In addition, the oncogenicity of retrovirally encoded variants of the c-Fos and c-Jun proteins has demonstrated a role for AP1 activity in cellular transformation. As a target for positive regulation by the mitogen-activated protein and Jun kinase cascades (35, 39), AP1 components have been implicated as downstream effectors in transformation by a number of oncogenes, and recent experiments have shown that transformation by the Ha-*ras* oncogene relies on the function of wild-type Jun (35, 37). Our results show that the DNs that inhibit AP1 DNA binding (A-Fos) are able to inhibit transformation better than DNs that are deleted for the transactivation domain (bZIP-Fos). The observation that A-Fos and bZIP-Fos have different effects on foci formation as compared with colony formation indicate that these reagents may be useful in unraveling the relationship between cell growth and transformation. Additional experiments in which stable cell lines expressing various levels of A-Fos are analyzed for growth and transformation properties will be necessary to establish the dose of A-Fos protein required to separate the biological effects of AP1 in mammalian cells (40).

Dominant negatives to the AP1 complex have been reported by several groups (41–44). These proteins act by interacting with endogenous AP1 family members and binding to DNA, which inhibits transactivation and transformation. A-Fos, the DN described in this manuscript, acts in a novel fashion; it heterodimerizes with endogenous AP1 family members, which inhibits DNA binding, thereby inhibiting transactivation and transformation. A-Fos, in both transactivation and transformation assays is more effective than bZIP-Fos or the Fos leucine zipper, the type of DNs used previously.

An additional advantage of the DN strategy presented here is the ability to explore repressive effects of a transcription factor. In different promoter contexts, some DNA-binding proteins can be either activators or repressors (41). The typical DN consisting of the bZIP domain could inhibit transactivation but not repression because the DNA site would remain occupied. Thus, previously described DNs are valuable for exploring the transactivation properties of a transcription factor but not the repression properties. The DNs described here should reveal any repressive properties of the AP1 complex in certain cell types and physiological conditions (40).

The experimental problem with these truncated proteins consisting of only the bZIP domain is the difficulty of demonstrating the mode of action. The DN to AP1 we have synthesized and characterized in this study forms heterodimers with Jun and prevents the normal AP1 family complex from binding DNA, creating a situation akin to ablating Jun family members' function in a cell. The inhibition of DNA binding should allow the demonstration in biological assays of the absence of DNA site occupancy using an *in vivo* footprint assay (25) and allow for the design of future experiments to identify target genes regulated by AP1.

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